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Full Length Research Paper

Physiological effect of the toxin from *Xanthomonas retroflexus* on redroot pigweed (*Amaranthus retroflexus*)

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A new toxin from *Xanthomonas retroflexus* could cause a series of physiological responses on seedlings of redroot pigweed. The experimental results revealed that respiratory ratio first increased 106% after treatment with the CFS (concentrated centrifugal fermentation supernatants) of *X. retroflexus* for 2 h, and decreased after treatment for 3 h; peroxidase (POD) activity increased 30% and 50% after treatment with CFS for 1 h and 2 h, respectively; Malon dialdehyde (MDA), soluble protein and mitotic indices appeared not to be affected by the toxin. Ultrastructure observation indicated that the thylakoids of chloroplast and cristae of mitochondria swelled, when the leaves were placed in the toxin for 2 h. After treatment with the phototoxin for 3 h, the cell membrane was disrupted, the chloroplasts disintegrated and the mitochondria vesiculated.

Key words: *Xanthomomas retroflexus*, toxin, redroot pigweed, weed control.

INTRODUCTION

About 30,000 kinds of weed are widely distributed in the world; 1800 of which cause yield losses every year that make up about 9.7% of total crop production. Weed control has always been an important aspect of environmental protection. Over the past century, chemical herbicides have been effectively employed to control various weeds. However, they may have caused many serious side-effects such as herbicide-resistant weed populations, reduction of soil and water quality, herbicide residues and detrimental effects on non-target organisms (TeBeest and Templeton, 1985; BeControlie and Morrison, 1993; Heap et al., 1993). With the heightening of the global environmental consciousness, bioherbicides especially microherbicides, which are highly effective for weed control and environmentally friendly as well have become very attractive both for research and for application.

Xanthomonas L4 with herbicidal activity was screened from the rhizosphere of redroot pigweed (*Amaranthus retroflexus* L.) using the high-throughput microscreen of Chlorella *pyrenoidosa* and the glutamine synthetase inhibitor model. According to analysis of its 16s rDNA sequence (GenBank accession number AY841369) similarity compared to that of *Xanthomonas campestris* pv. Campestris and *Xanthomonas* LA37, *Xanthomonas* L4 was named *Xanthomonas retroflexus* (Ming-Zhi Li et al., 2004). Structure elucidation indicated that the toxin is composed of minor molecular compounds including six organic acids and cyclo-(proline-phenylalanine) (Ming-Zhi Li et al., 2004); further experiments indicated that it was very stable under high temperature and has the highest bioactivity at pH 4. The experimental results from Petri dish bioassays in the laboratory and weed-soil bioassays in the greenhouse demonstrated that the *X. retroflexus* had effective control ability for broad leaf weeds such as

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redroot pigweed and shepherdspure (*Capsella bursa-pastoris*) (Li Yong-Quan et al., 2003).

This research work reports the physiological effect of the toxin from *X. retroflexus* on redroot pigweed; especially effects on the respiratory ratio, POD, MDA, soluble protein, cell division and ultrastructure of the leaf.

MATERIALS AND METHODS

Cultivation of seedlings

The seeds of redroot pigweed were randomly selected, sterilized with 0.01% NaOCl for 1 min followed by 70% ethanol for 1 min, and then washed in distilled water. Twenty seeds were placed in a Petri dish and germinated at 25-26℃ with 50%-60% humidity in an artificial intelligence incubator for 16 h in light followed by 8 h in darkness. After 5 days of incubation, uniform shoots for use in experiments were selected.

20 seeds of redroot pigweed were grown in pots filled with ordinary soil inside the greenhouse in daylight with temperatures between 20 and 25℃ until 4-6 leaf age.

Centrifugal fermentation supernatants (CSF) preparation

X. retroflexus was fermented on a rotary shaker at 210 rpm and 28℃ in bacterial medium (beef extract 5.0 g, peptone 10.0 g, NaCl 15.0 g, dH2O 1000 ml) for 72 h. The CFS broth was centrifuged with 4000×g for 15 min and then condensed to 1/2 volume using a vacuum evaporative method.

Respiratory ratio assay

Respiratory ratio of the 3rd leaf of redroot pigweed seedling was measured with L1-6400 Portable Photosynthetic System (L1-6400, LI-COR, Lincoln, USA) after spraying with CFS for 1.2, 1.5, 2, 3 and 4 h. The assay was carried out in an open condition with 420±27 ppm of CO2 in the leaf chamber, 500 ml/s of airflow at 23±1℃ and 21±2% of humidity. Three replicates per treatment were used and analyzed with Duncan`s test.

Observation of cell ultrastructure

After spraying CFS to 4-6 leaf age redroot pigweed for 1, 2, 3 and 4 h, treated leaves were picked and fixed with 2.5% glutaraldehyde for 24 h, rinsed several times with 0.05 M pH 7 phosphate buffer, post-fixed 3 h with OsO4, rinsed with 0.05 M phosphate buffer for 2 h, dehydrated in a gradient alcohol series, and embedded in Spurr`s resin. Ultrathin sections were stained with uranyl acetate and lead citrate; cell ultrastructure was observed with Mitachi-800 (Qian Yang, 1994; Laberge and Rouillon, 2000; Fayez, 2000).

POD, MDA and soluble protein assay

After spraying CFS on the 4-6 leaf age redroot pigweed at room temperature for 1 h, 2 h, 3 h and 5 h, respectively, 1 g sample of treated leaves for each group was rubbed with 5 ml of 0.02 mmol/L KH2PO4 for 5 min, and then centrifuged at 4000×g for 15 min to get the supernatant. 1 ml of supernatant was mixed with 3 ml of the reaction solution (50 ml of 0.1 mmol pH 6.0 H3PO4, 28 µl of guaiane, 30% H2O2), in which guaiane was oxidated by POD (peroxidase) to become brown matter and turned brown. POD activity was determined specifically with guaiacol at 470 nm using the Jung Sunyo's method (Sunyo, 2004).

MDA activity was determined as followed after spraying CFS on 4-6 leaf age redroot pigweed for 2, 3 and 5 h at room temperature. 0.35 g of treated leaves of each group were rubbed with 5 ml H2O, mixed with 5 ml 0.5% of thiobarbituric acid, seethed for 10 min, centrifugated with at 4000×g for 15 min. The MDA (malon dialdehyde) in supernatant was measured at 532 nm with a 6010-ultraviolet spectrophotometer (Jianming and Chunrong, 2002).

Total protein concentration was determined by the method of Bradford (1976), all enzyme activities and protein concentrations in the treated seedlings were expressed as a percentage of corresponding values for the untreated plants.

Observation of cell division

The tips of treated redroot pigweed root were fixed in Carnoy fluid for 24 h, hydrolyzed for 15 min in 1 N HCl, and stained for 10-15 min with carbol fuchsin. The 1 mm tip segment of each stained root was excised and observed under a light microscope at 400 X magnifications. Mitotic indices were determined by counting the number of division figures per 1000 cells of each meristem (Chauhan and Saxena, 1998).

Statistical analysis

All experiments were set up as completely random designs. Statistics were conducted using a mean three repeat test based on Dunnett's LSD and calculated with exact 95% confidence intervals ($P<0.05$) using the binomial distribution.

RESULTS

Respiratory ratio

After treatment with this toxin for 1.2, 1.5 and 2 h, the respiratory ratio of leaves was increased by 66, 47 and 106%, to reach 1.61, 1.6343 and 1.8377 µmol/m2s1, respectively. However, when the leaves were treated for 3 and 4 h, the respiratory ratio of leaves dramatically decreased to 1.5567 and 1.1567 µmol/m2s1, respectively. The leaves wilted simultaneously, but the respiratory ratio of control increased to 1.3243 and 1.7143 µmol / m2s1 using the same apparatus repeatedly (Figure 1), which is due to contamination with the toxin. Treated tests and the control test had showed significant differences of 5% with Dunnett's test, which demonstrated that L4 toxin, can induce increase of respiratory ratio in a short time.

Observation of cell ultrastructure

When the seedlings were treated with the toxin of *X. retroflexus* for 1 h, the chloroplast distorted although the thylakoids were still seen clearly, and the outer membrane of the mitochondria was also disintegrated. When the leaves suffered the poison of the toxin for 2 h, the thylakoids of the chloroplast and the cristae of the mitoch-

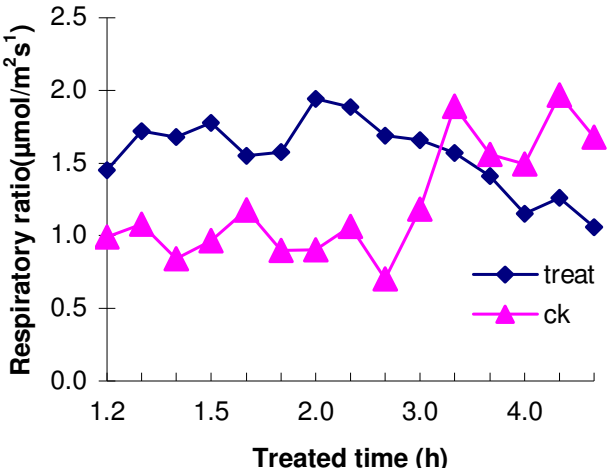

Figure 1. Effect of the toxin on the respiratory ratio of Redroot pigweed.

ondria swelled. After treatment with poison for 3 h, cell membranes disrupted, the chloroplast swelled and the mitochondria vesiculated. Furthermore, the cristae and the inner membrane of the mitochondria disintegrated (Figure 2).

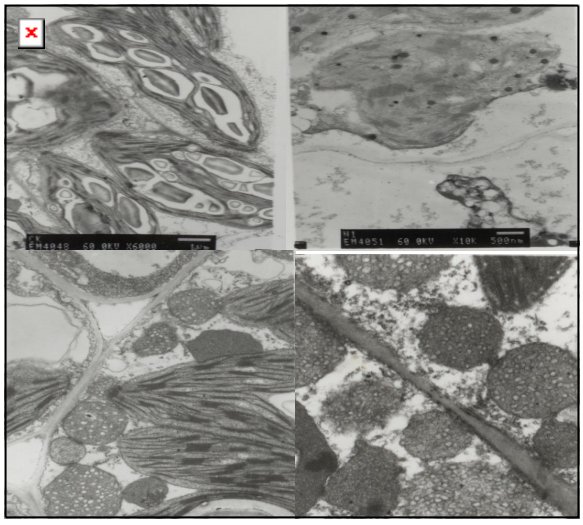

Figure 2. Transmission electron micrographs of chloroplast ultrastructre and mitochondria after treatment with the toxin. **a.** Control. **b.** Chloroplast ultrastructre (5000 magnification) after treatment for 3 h. **c.** Control. **d.** Disintegrated outer membrane of mitochondria (5000X magnification) after treatment for 1 h.

POD, MDA and total soluble protein activity

The experimental results of POD are shown in Figure 3, after treatment with the toxin for 1, 2 and 3 h. The experimental results indicated that POD activity increased by

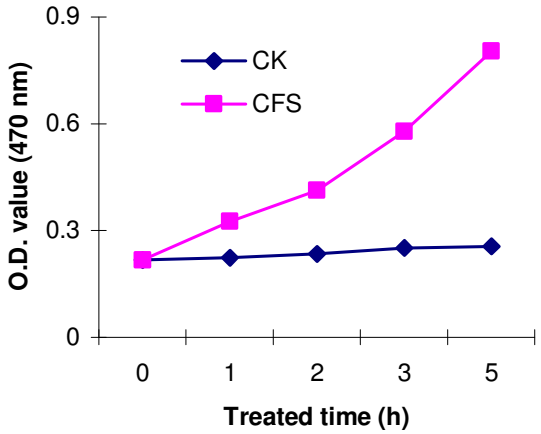

Figure 3. Effect of the toxin on POD activity of redroot pigweed.

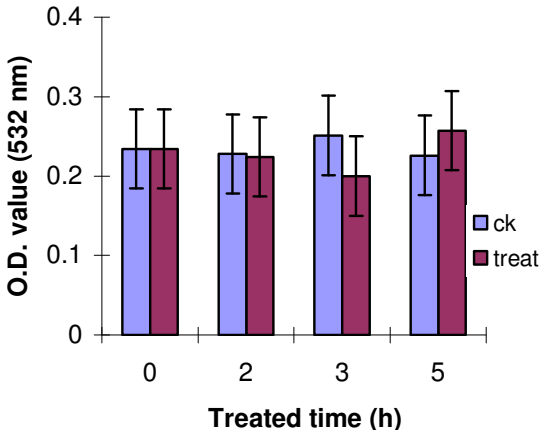

Figure 4. Effect of the toxin on MDA activity of redroot pigweed.

by 45, 68 and 131%, respectively, compared to the control, which was significantly different at P0.05 level. POD is a kind of multifunctional enzyme which can turn maleficence metabolic products into harmless substances. It facilitates transformation of intermediate metabolites, thus having a positive effect on plant growth. Once the seedlings are infected by the toxin, the POD in plant cells will increase for self-adjustment under adversity.

The change of MDA activity is shown in Figure 4 after treatment with the toxin for 2, 3 and 5 h at room temperature. The experimental results indicate that MDA activity is not significantly different at P0.05 level. Though POD activity obviously increased, MDA activity did not increase. Thus, the *X. retroflexus* toxin did not cause the peroxidation reaction of membrane lipids, because MDA is a product of the peroxidation reaction.

After treating with CFS for 3, 6 and 22 h, the OD595 value of soluble protein of redroot pigweed leaves was 0.564, 0.559 and 0.612, respectively (Figure 5). The data

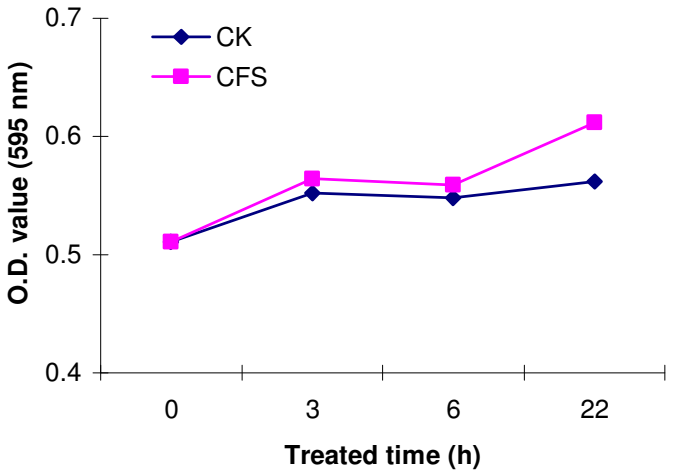

Figure 5. The effect of the toxin on soluble protein synthesis of redroot pigweed.

showed no significant difference compared to the control with Dunnett's test (P>0.05). So the conclusion was reached that *X. retroflexus* toxin has little inhibitory effects on protein synthesis.

Observation of cell division

The effect of the *X. retroflexus* toxin on cell division was determined from quantification of mitotic indices, which was performed by using root meristems of redroot pigweed treated with 1:1 CFS. The experimental results indicated that there was no significant difference (P>0.05) using statistical analysis of mitotic indices (Table 1), and the mitotic index (77 and 69 division figures per 1000 cells) was similar to that of control (76 and 80 division figures per 1000 cells) after treatment for 12 and 24 h, respectively, even though *X. retroflexus* toxin reduced root elongation above 90% after treatment with 1:1 CFS for 24 h. Therefore, the *X. retroflexus* toxin did not inhibit nuclear division.

DISCUSSION

Different herbicides have different mechanisms of weed control, and toxin always show complicated action mechanisms. Generally, herbicides inhibit the mitosis, respiration and photosynthesis of weeds, or disrupt cell membranes directly. For example, Dehydrozaluzanin C causes rapid leakage of the plasma membrane, but photosynthesis, respiratory and mitotic processes appear to be unaffected (Galindo and Hernandez, 1999). Shabana et al. (2001) reported that the specific growth ratio, cell number, chlorophyll level and dry weight yield of the green algae decreased significantly with increasing pendimethalin concentrations, while protein and carbohydrate contents increased significantly; on the other hand, photosynthetic activity decreased whereas dark respiretion increased with high pendimethalin concentrations (Shabana et al., 2001). Macrocyclic trichothecene toxins produced by *Myrothecium verrucaria* were tested for controlling duControlweed (Lemna pausicostata L.) by plantlet cultures and kudzu (Pueraria lobata L.) leaf disc assays. Rordin and verrucarin derivatives from M. verruccaria showed high phytotoxic activity in decontrol weed, causing an increase of cellular leakage, growth inhibition and chlorophyll loss with increasing concentration to 10 µm (Abbas and Johnson, 2002).

However, the toxin from *X. retroflexus* can increase respiratory ratio. Respiration is an important metabolic process, which reflects the plants' adaptability to damage. When the seedlings were treated with the toxin continuously, the respiration was increased and other inner recovery systems were also redeployed. However, excessive respiration consumes excessive inner energy, water and substance. At the same time, the inner membrane of mitochondria was also disrupted and ATP could not be synthesized, at last resulting in wilting of the weed. It could be deduced that the oxidative phosphorylation was uncoupled when the mitochondria were disrupted by *X. retroflexus* toxin.

Eutypine, a toxin from Eutypa lata, caused a large increase of respiration in the concentration range of 0-150 µm and a subsequent decrease of both respiratory control and ADP/O ratio. The experimental results showed that eutypine might act on mitochondria, particularly on its inner membrane by uncoupling mitochondrial oxidative phosphorylation in grapevine and potato cells (Deswarte and Canut, 1996; Deswarte et al., 1996). So the action mechanism of *X. retroflexus* toxin on redroot pigweed is very similar to that of eutypine.

Table 1. The effect of the toxin on root cell division of redroot pigweed.

Treated time	Mitotic index	
	Control	CFS 1:2
12 h	7.5	7.67*
24 h	8.06	6.85 *

*Statistically lower than the control at the 0.05 level using Dunnett's test.

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